

-1-

Date: August 4, 2000 Express Mail Label No. EL551543089US

Inventors: Barbara A. Gilchrest, Mina Yaar and Mark Eller  
Attorney's Docket No.: 0054.1087-010

## METHODS OF INDUCING HAIR GROWTH AND COLORATION

### RELATED APPLICATIONS

This application is a Continuation of PCT/US99/02362 filed February 3, 1999,  
which is a Continuation-in-Part of U.S. Serial No. 09/018,194 filed February 4, 1998,  
which is a Continuation-in-Part of U.S. Serial No. 08/793,683 filed <sup>April 3, 1997 (abandoned)</sup> August 30, 1995,  
which is the U.S. National Phase of PCT/US95/10971 filed 8/30/95, which is a  
Continuation of U.S. Serial No. 08/298,941, filed 8/31/94. <sup>(Patent No. 6,103,692)</sup> The entire teachings of each  
of these applications are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

- 10 Normal hair follicles cycle between a growth stage (anagen), a degenerative stage (catagen), and a resting stage (telogen). The scalp hairs have a relatively long life cycle: the anagen stage ranges from two to five years, the catagen stage ranges from a few days to a few weeks, and the telogen stage is approximately three months (Fitzpatrick, T.B., *et al.*, eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I),
- 15 McGraw-Hill, Inc., 1993, pp. 290-291; Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), pp. 1-17 (1991)). Shorter hairs found elsewhere on the body have corresponding shorter anagen duration. The morphology of the hair and the hair follicle changes dramatically over the course of the life cycle of the hair.

- During anagen, the hair follicle is highly active metabolically (Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), p. 4 (1991)). The follicle comprises a
- 20 follicular (dermal) papilla at the base of the follicle; epidermal matrix cells surrounding

R monoclonal antibody believed to act as a pseudo-ligand for the p75 NGF-R. (Anti-human p75 NGF-R monoclonal antibody courtesy of Moses V. Chao, Cornell University Medical Center, New York, NY; Ross, *et al.*, Proc. Natl. Acad. Sci. 81:6681 (1984)). Like NGF, the antibody suppressed melanocyte apoptosis in UV-irradiated  
 5 cultures, while anti-rat p75 NGF-R antibody that did not bind the human p75 NGF-R had no effect.

Northern blot analysis of melanocyte RNA from donors of different ages showed that p75 NGF-R was higher in older donors, while in contrast the level for other growth factor receptors was unchanged or decreased with age, suggesting a greater  
 10 vulnerability to apoptosis with aging, consistent with the clinical tendency for older persons to experience progressive hair loss.

Thus, one embodiment of the present invention relates to a method of preventing or inhibiting melanocyte cell loss after injury. The melanocytes are located in the basal epidermal layer and include melanocytes located in the skin and in hair follicles. The  
 15 type of injury includes injury due to exposure to ultraviolet light, especially UVB, for example, in habitually sun-exposed skin, and injury due to the normal aging process. Injuries can also include disease conditions such as alopecia areata, telogen effluvium, and androgenic alopecia. The treatment of male-pattern baldness is also encompassed by the present invention.

20 More specifically, the invention relates to methods of preventing, or inhibiting, apoptosis in melanocytes and keratinocytes. As described above, Applicants have shown that apoptosis in melanocytes is mediated by the p75 NGF receptor. If the receptor is occupied, that is, if the receptor has bound an appropriate ligand, apoptosis is inhibited in the cell. Examples of appropriate ligands include neurotrophins, nerve  
 25 growth factor, biologically active fragments of neurotrophins and NGF, such as the NGF 26kD  $\beta$ -subunit, and peptides or other small molecules that mimic the region of neurotrophins and NGF that bind to the p75<sup>NTR</sup>, also referred to herein as p75 psuedo-ligands. Such psuedo-ligands include small peptides such as the cyclic peptide, CATDIKGAEC<sup>(SEQ ID NO: 9)</sup> described herein. The psuedo-ligands of the present invention bind to

B

The chimeric antibodies can comprise portions derived from two different species (e.g., human constant region and murine variable or binding region). The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as single contiguous proteins using genetic engineering techniques. The portions derived from two different species can also be produced by recombinant means and then joined as described above. DNA encoding the proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed as contiguous proteins or can be produced by recombinant means and joined using techniques known to those of skill in the art.

One mechanism by which p75 induces apoptosis may involve the cellular ratio of p75<sup>NTR</sup> to TRK in combination with the level of NGF. The abundance of NGF, the neurotrophin that presumptively enhances the survival of cells that express both of its receptors, decreases with age (Larkfors, L. *et al.* Brain Res. 1987; 427:55-60). In addition, the Applicants have found a prominent increase in the expression of the apoptotic p75<sup>NTR</sup> with aging (Yaar, M. *et al.*, J. Invest Dermatol., 1997;108:568). These data suggest that a relative lack of NGF and/or increased levels of an apoptosis-inducing ligand such as beta-amyloid may lead to apoptotic loss of cells. Furthermore, as described herein ligand binding to the p75<sup>NTR</sup> resulted in receptor aggregation, which led to the initiation of the apoptotic pathway. Psuedo-ligands that bind to the p75<sup>NTR</sup> but do not aggregate the receptor, such as the cyclic peptide CATDIKGAEC<sup>(SEQ ID NO: 9)</sup>, can inhibit apoptosis.

The end result of p75 NGF-R and trk binding to its ligand is the expression of the protective protein, Bcl-2. Bcl-2 has been shown to prevent some classes of cell death in lymphocytes and neurons. (Veis, D.J., *et al.*, Cell 75:229- 240 (1993)). As described in Example 4, Applicants have now shown the expression of Bcl-2 by injured melanocytes after treatment with NGF. Apoptosis can be inhibited by the expression of the protective protein, Bcl-2. Thus, another method of preventing melanocyte cell loss comprises a method of upregulating expression of the Bcl-2 protein in melanocytes. This can be accomplished, for example, by inserting a nucleotide sequence encoding

0054.1087-010

B

observed at the expected 25 nM cyclic peptide concentration. This experiment demonstrates the cyclic peptide can compete with beta-amyloid 1-40 for binding to the p75<sup>NTR</sup> receptor.

#### EXAMPLE 18: EFFECT OF KGA-CONTAINING PEPTIDES ON CELL SURVIVAL

- 5 p75<sup>NTR</sup>-NIH 3T3 cells were maintained in DME supplemented with 10% calf serum until 80% confluent. The cells were washed and incubated in serum free DME containing transferrin (5 microg/ml) and insulin (5 microg/ml). Triplicate dishes were supplemented with diluent or preaggregated beta-amyloid 1-40 (250nM) as positive and negative controls, respectively. Figure 12A shows the results from triplicate dishes
- 10 supplemented with cyclic decapeptide CVGSNKGAIC (250nM, SEQ ID NO:4) alone or together with preaggregated beta-amyloid 1-40 (250nM). Figure 12B shows the results from triplicate dishes supplemented with cyclic 250nM decapeptide CATDIKGAEC (SEQ ID NO:9) alone or together with preaggregated beta-amyloid 1-40 (250nM). Figure 12C shows the results from triplicate dishes supplemented with
- 15 250nM cyclic hexapeptide CKGAIC (SEQ ID NO:10) alone or together with preaggregated beta- amyloid 1-40(250nM). After 72 hours, cells were rinsed in PBS and cultures were incubated in 0.25% trypsin at 37°C. Cell yields were determine using a particle counter. Cell yields in cultures supplemented with each cyclic peptide and beta-amyloid were significantly higher than cell yields of cultures supplemented with
- 20 beta-amyloid alone ( $p < 0.01$  CVGSNKGAIC;  $p < 0.0002$  CATDIKGAEC;  $p < 0.002$  CKGAIC; non-paired t test comparing the effect of beta-amyloid and peptide to beta-amyloid alone. Test was performed separately for each group).

#### EXAMPLE 19: ELUCIDATION OF APOPTOTIC SIGNALING PATHWAYS FOLLOWING ACTIVATION OF THE 75 KD NEUROTROPHIN RECEPTOR.

- 25 The 75 kD neurotrophin receptor (p75) is strongly expressed in keratinocytes, melanocytes and neurons and has been implicated in apoptosis of these cells under certain conditions. When neurotrophins activate p75 together with receptors of the Trk

family, p75 evokes a survival signal. However, when p75 is activated alone, it may signal for apoptosis by stimulating within minutes sphingomyelin turnover and ceramide generation. Still, the sequence of events linking p75 stimulation to ceramide generation and apoptosis remain largely unknown.

- 5 To investigate p75 early signaling, NIH-3T3 cells engineered to constitutively express human p75 (3T3-p75), were stimulated with a known p75 ligand  $\beta$  amyloid ( $\beta$ A), and the distribution of p75 on the cell surface was analysed using immunohistochemistry and confocal laser microscopy. Within minutes  $\beta$ A-treated cultures displayed aggregation of p75, while the baseline, homogeneous cell surface
- 10 distribution of p75 did not change in diluent treated cultures. Furthermore, 3T3-p75 stimulated with  $\beta$ A in the presence of a bifunctional crosslinker and then reacted with anti p75 antibodies displayed on western blots in addition to the expected 75 kD band also a ~220-230 kD band, consistent with receptor trimerization, as reported for other apoptotic signaling pathways. Moreover, similar to signaling initiated by the apoptotic
- 15 TNF- $\alpha$  and Fas receptors,  $\beta$ A activation of p75 strongly induced the transcription of the immediate early c-jun mRNA, stimulated the stress-activated c-Jun NH<sub>2</sub>-terminal kinase (JNK) as measured by phosphorylation of its substrate [GST-cJun (1-79)], activated caspase-3 to cleave its substrate [poly-(ADP ribose)polymerase], and induced the characteristic DNA fragmentation into multimers as measured by TUNEL analysis and
- 20 DNA ladder formation. (Figure 13)

To determine if the initial step of p75 aggregation is required for initiation of apoptosis, 3T3-p75 were pretreated with an HPLC purified cyclic peptide (CATDIKGAEC)<sup>(SEQ ID NO: 9)</sup> that binds the ligand binding site of p75, and then cultures were stimulated with  $\beta$ A or with diluent alone. The cyclic peptide inhibited p75 aggregation,

25 decreased c-jun transcription that was otherwise prominent in UV-irradiated diluent-treated keratinocytes. These data identify for the first time the initial signaling events that follow p75 activation and suggest that signaling through p75 requires receptor aggregation.

# EXAMPLE 20: REGULATION AND ACTIVATION OF THE 75 KD NEUROTROPHIN RECEPTOR IN HUMAN MELANOCYTES.

Melanocytes (MC) express the 75 kD neurotrophin (NT) receptor (p75) that binds all Nts. In the presence of Trk receptors, NTs bind both p75 and Trk and signal through Trk. However, data suggest that even when p75 is present alone, it may be activated to signal cell survival or apoptosis. Because in neural crest cells p75 expression is down-regulated by increased cyclic AMP (cAMP) levels that occur after nerve injury, it was investigated whether p75 is similarly regulated in MC.

MC were stimulated with growth factor-containing medium supplemented with forskolin (50 $\mu$ M) or IBMX (100 $\mu$ M) that increase cAMP levels. In diluent treated cells, p75 mRNA increased within 24 hours but forskolin and IBMX substantially inhibited this upregulation. To determine if ultraviolet (UV) irradiation that induces cutaneous injury upregulates p75, MC were sham- or UV-irradiated with solar simulated light (30 mJ/cm<sup>2</sup>, metered at 285+5nm). After an initial down-regulation within 4 hours, at 24 and 48 hours p75 mRNA was strongly induced in UV- vs sham-irradiated cells. Furthermore, 48 hours after irradiation, cAMP levels were > 70% decreased in UV- vs sham-irradiated cells.

To investigate p75 signal transduction, it was first determined by RT-PCR that trkA and trkC are not expressed in MC maintained in choleraen/TPA-free medium. MC were incubated with 100 ng/ml nerve growth factor (NGF) or neurotrophin-3 (NT-3) for 30 minutes and c-Jun amino terminal kinase (JNK) activation was determined. Compared to diluent or NT-3, NGF substantially induced phosphorylation of GST-c-Jun(1-79). Moreover, pre-incubation of MC with a cyclic peptide CATDIKGKEC<sup>(SEQ ID NO: 9)</sup> that binds p75 abrogated JNK activation in NGF stimulated cells. Furthermore, within 3 hours p75 activation by NGF, but not by NT-3, lead to a 2-fold increase of intracellular ceramide. As before, ceramide increase was abrogated by pre-incubation with the cyclic peptide. Because in cell lines, UV irradiation directly activates cell surface receptors such as Fas and the TNF $\alpha$  receptor, sham- or UV-irradiated MC were used to determine p75 activation. Within 30 minutes